THE EFFECT OF CHRONIC EXPOSURE TO FLUORINE AND 7,12-DIMETHYLBENZ(A)ANTHRACENE ON ANXIETY, LOCOMOTOR ACTIVITY, SPATIAL LEARNING, AND MEMORY CONSOLIDATION IN RATS

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ABSTRACT: In this study, the effect of fluorine, which is known to cause chronic toxication, and 7,12-dimethylbenz(a)nthracene (DMBA) on anxiety, locomotor activity, spatial learning, and memory consolidation in rats were investigated. Ninety adult Wistar albino male rats, weighing 150–200 g, were divided into 9 groups of 10 animals and treated for 90 days with fluoride (F), the ion of fluorine, in drinking water in the form of sodium fluoride (NaF) and weekly oral DMBA (10 mg/kg bw/po/weekly) in a sesame oil vehicle, in the following manner: group 1: 0 ppm F, 0 mg/kg bw DMBA (control); group 2: sesame oil weekly (vehicle control); group 3: 1 ppm F; group 4: 15 ppm F; group 5: 30 ppm F; group 6: DMBA; group 7: 1 ppm F and DMBA; group 8: 15 ppm F and DMBA; and group 9: 30 ppm F and DMBA. Tests for anxiety (elevated plus maze), locomotor activity (rotarod), and spatial learning and learning consolidation (Morris water maze) were administered to rats at the beginning and at the 30th, 60th, and 90th days of the study. The results showed that anxiety was increased by F at a low dose and DMBA, and decreased by high dose F. Locomotor activity was increased by F and decreased by F+DMBA. DMBA could either increase or decrease motor activity depending on the time of assessment. Spatial learning and memory consolidation were decreased by both F and DMBA. After considering these results, we plan to conduct further studies to clarify the mechanisms underlying these neurotoxic effects of fluorine and DMBA.

Keywords: Anxiety; DMBA; Fluorine; Fluorosis; Locomotor activity; Memory consolidation; Spatial learning.

1. INTRODUCTION

Some of the elements in rocks, groundwater, and soil are present naturally, or as a result of industrial use, at levels that may have negative effects on human health. One such element is fluorine which is found in soil, water, rock, and air as well as in plant and animal tissues. It has an atomic weight as 18.998, an atomic number of 9, a molecular weight as 38, and a valance number of 1. It forms 0.027% of the earth's crust.[1]

Fluoride, the ion of fluorine, may be present at higher levels in spring waters and plants where volcanic soils are found.[2] If the amount of fluorine taken daily exceeds the safety threshold, chronic fluorine intoxication known as fluorosis occurs. As a result of fluorosis, pathological changes may occur in the teeth, skeletal system, liver, kidney, heart, gastrointestinal tract, nervous system, and endocrine system.[3] According to World Health Organization (WHO) data, 1.5 ppm is considered to be the upper safe limit for fluoride drinking water but that lower Country Standards may be appropriate, such as the standards for India of 1 mg/L, with the rider that the "lesser the fluoride the better, as fluoride is injurious to health," and Senegal, West Africa, of 0.6 mg/L.[4] Worldwide the countries affected by endemic fluorosis due to excess fluoride in drinking water, occurring naturally or a result of adding fluoride, include Algeria, Argentina, Australia, Bangladesh, China, Egypt, Ethiopia, India, Iran, Iraq, Japan, Jordan, Kenya, Libya, Mexico, Morocco, New Zealand, Palestine,

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Pakistan, Senegal, Sri Lanka, Syria, Tanzania, Thailand, Tunisia, Turkey, Uganda, and United Arab Emirates.[5-6]

Fluorosis causes acute or chronic symptoms according to the duration and amount of intake.[7] Oxidative stress caused by fluorosis may cause tissue and organ damage.[8] A chronic fluorine intake at high dosage may cause neural damage[9] by triggering neurodegenerative alterations and thus attenuating learning and memory, causing abnormal behavior, and causing a degeneration in the body physiology.[10] As a matter of fact, there is a decrease in the development of intelligence (IQ) in children in regions with high levels of fluorine. It has been suggested that fluorine affects cognitive functions and memory negatively by affecting protein and enzyme systems.[11] Experimental studies have also shown that chronic fluorosis induces apoptosis in brain neurons.[12]

Today, although rapidly developing industrialization makes human life considerably easier, it also brings with it many environmental problems. Humans are exposed to toxic and carcinogenic substances by inhaling polluted air or consuming contaminated water or food. Industrial wastes, pesticides, cigarette smoke, and chemicals emitted from environmentally harmful substances, such as industrial flue gases, may be incorporated into air, water, soil, and food and cause a threat to human health. The polycyclic aromatic hydrocarbon (PAH) derivative 7,12-dimethylbenz(a)anthracene (DMBA) contained in these substances has a toxic and carcinogenic effect for human health.[13] DMBA plays an important role in the formation of cancer, both by direct interaction with DNA through its metabolites and by the oxidative stress it generates.[14] PAH compounds need to undergo metabolic reactions in order to produce a mutagenic and carcinogenic effect.[15] PAHs are sometimes transformed into reactive intermediates as a result of metabolism and these products are covalently bound to nucleic acids and gain genotoxic properties.[16] Different studies have shown that PAH derivatives cause neurological problems such as memory loss, cognitive impairments, such as learning difficulties, and parasympathetic imbalances by crossing the blood-brain barrier.[17,18] Cognitive function disorders due to PAH derivatives are suggested to be related with monoamine, amino acid, and choline deficiency[19] or may be due to alterations in the expression of NR1 and NR2A which are NMDA glutamate receptors in the brain region which is responsible for memory and anxiety.[20,21] People and animals living in volcanic areas, which are natural sources of PAHs, are exposed to high levels of fluorine and also to polycyclic aromatic hydrocarbon derivatives. Yildirim et al.[22] reported that changes in the blood and oxidative stress parameters and in the hepatic, renal, and cardiac histopathology play an important role in the physiopathology of the toxicity induced in the liver, kidney, and heart tissues by NaF, DMBA, and NaF+DMBA.

In the literature reviews, there is no study about the neurological effects of chronic exposure to polycyclic aromatic hydrocarbon derivatives in association with fluorine. Therefore, in this study we aimed to evaluate, experimentally in rats, the effects of fluorine and 7,12 dimethylbenz(a)anthracene, a polycyclic aromatic hydrocarbon derivative, both alone and in combination on anxiety, locomotor activity, spatial learning, and memory consolidation.

2. MATERIALS AND METHODS

2.1. Animals: All the experiments were performed on 90 adult male Wistar albino rats weighing 150–200 g from the animal experiment center of Ankara Başkent University. The animals were housed in a well-ventilated and air-conditioned area provided with independently adjustable light-dark cycle (12 hr light/12 hr dark cycle) and temperature regulation systems. Temperature was maintained at 22±2ºC and humidity was kept at 45–70%. The rooms and animal cages were cleaned daily and the animals were provided with fresh food and water *ad libitum* on a daily basis. This study was approved by Van Yüzüncn Yıl University Experimental Animals Local Ethics Committee (YUHADYEK 02.03.207/ Decision no: 2017/02).

2.2. Chemicals: The chemicals used were 7,12 dimethylbenz(a)anthracene (Sigma-D3254), NaF (Sigma-S7920), fluorine standard solution (Metter Toledo), TISAB 2 solution (Metter Toledo), and ketamine hydrochloride (Ketalar, Pfizer).

2.3. Groups: Ninety adult Wistar albino male rats, weighing 150–200 g, were divided into 9 groups of 10 animals and treated for 90 days with fluoride (F), the ion of fluorine, in drinking water in the form of sodium fluoride (NaF) and weekly oral DMBA (10 mg/kg bw/po/weekly) in a sesame oil vehicle, in the following manner: group 1: 0 ppm F, 0 mg/kg bw DMBA (control); group 2: sesame oil weekly (vehicle control); group 3: 1 ppm F; group 4: 15 ppm F; group 5: 30 ppm F; group 6: DMBA; group 7: 1 ppm F and DMBA; group 8: 15 ppm F and DMBA; and group 9: 30 ppm F and DMBA.

On the 30th, 60th, and 90th days of the study, the rats were subjected to anxiety tests (elevated plus maze test), motor activity tests (rotarod), and learning tests (Morris water tank test). Blood samples were collected from the rats by intracardiac method under ketamine anesthesia for the analysis of fluorine levels in the serum after the completion of all the tests. The sera obtained by centrifugation were maintained at –80ºC until the fluorine analysis.

2.4. Serum fluorine analysis: During measurement of the fluorine ion activity, the total ionic strength adjustment buffer (TISAB) was used to keep the total ionic strength of the solution constant, to adjust the pH, and to degrade the fluorine ion complexes with metal cations such as aluminum, iron, and magnesium. Blood samples taken from rats were centrifuged at 4ºC, 3,000 rpm for 15 minutes, and serum was removed. One mL of TISAB II solution was then added and the combined pH and selective fluorine electrode was immersed in the mixture to check that the pH was between 5.0 and 5.5. The fluorine measurement, in ppm, was taken when the reading was stable.[23]

2.5. *Elevated plus maze test:* The elevated plus maze test was started in the laboratory of the Van Yüzüncn Yıl Faculty of Medicine, Department of Pharmacology, prior to the application of chemical substances on the first day of the experiment. Then, at 4, 8, and 12 weeks, the test was repeated. The animals were brought to the laboratory at least 3 hours before the test for acclimatization. To minimize the anxiety of the animals, the maze was assembled in an isolated room away from any external interference by noises, scents, or movement. A plus-shaped (+) maze, elevated 50 cm from the ground, was used consisting of two opposite open and two opposite closed arms (width 12 cm, length 50 cm) and a central square. Each

rat was placed in the central square of the maze facing one of the open arms and was video recorded for 5 min using the Noldus Ethno Vision Tracking System. The maze was cleaned after the testing of each animal. The proportion of entries into open arms/total entries and the percentage of time spent on the open arms/total time were calculated.[24,25]

2.6. Morris water maze test: Morris water maze (MWM) test was performed using the spatial version of the MWM test utilized by Tuzcu and Baydaş.[26] For the pretraining orientation, the rats were made to swim in the platform-free maze for 2 min. Before the testing, environmental cues that aid the rats in spatial learning including high-contrast geometric patterns were placed on the walls visible to the animal from the water and platform throughout the duration of the experiment. Using a computer equipped with the Noldus EthoVision Tracking System, the maze was divided into 4 equal quadrants. Care was taken to position the platform in the same quadrant throughout the test. Each rat swam 4 times within four consecutive days. The time spent in locating the platform (reaction time) and the time spent in the platform quadrant were recorded for each rat.

Each rat was allowed to swim for a maximum period of 90 sec.[27] The rats that could not locate the hidden platform during this period were guided to the platform by the researcher. For such cases, the reaction time was accepted as 90 sec. During both the training and testing sessions, the rats were left on the hidden platform for a brief period of 30 sec in order to allow them to recognize the environmental cues. To avoid rote learning, the rats were placed in a different starting point prior to each testing session. Care was taken to use the same starting points for each rat in the same order.[28]

At the testing at 90 days, on the four experimental days of testing, each rat was made to swim in the platform-free maze for 1 min in order to determine the reference memory. The percentage of the time spent in the platform quadrant was accepted as the indicator of memory retention.

2.7. Rotarod test (motor activity test): In the rotarod test, the walking period of the test animal is measured on a rotating rod at a constant speed. In the first phase of the study, the rats were trained on the device for 3 minutes at 6 rpm without falling. Each animal received three trials of 60 sec at a speed of 16 rpm. In this way, the total score was shown as the sum of the three trials (maximum value 180 sec). The time (sec) spent by the rats on the device without falling was considered as the rotarod performance.[29,30]

2.8. Statistical analysis: In this study, analyzes were performed by using SPSS (version 21) statistical software program. For continuous variables in the study, the mean and standard error values were calculated. The Kruskal-Wallis test was used to compare the differences between the groups and the controls in terms of the measured parameters. Differences between measurements in the same group were calculated by using the Friedman Test. The statistical significance criterion was accepted as 5% in the comparison and all parameters were evaluated accordingly.

3. RESULTS

This study was completed in 90 days. Anxiety due to fluorine and DMBA was evaluated with the elevated plus maze test. The effects of these chemicals on spatial

learning and memory consolidation were assessed with the Morris water tank test. In addition the rotarod test was performed for the evaluation of locomotor activity.

3.1. Serum fluorine levels: The results of the fluorine analysis of the serum samples of the rats taken on the 90th day are shown in Figure 1.

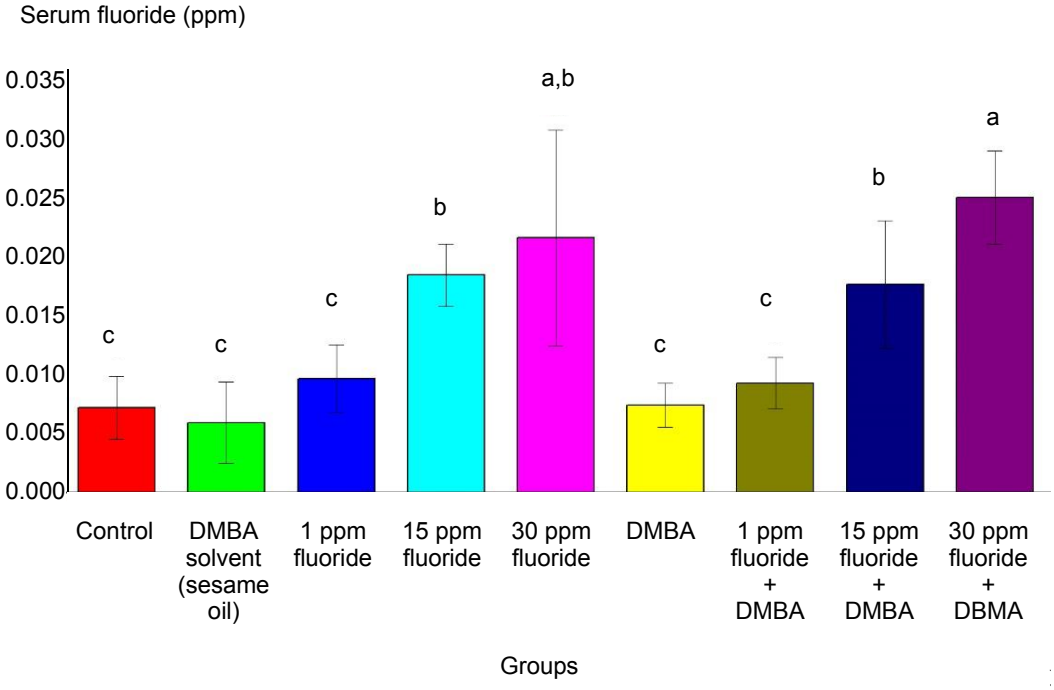

Figure 1. Serum fluorine levels (ppm) in the various groups given the DMBA solvent (sesame oil), fluoride in the drinking water, or weekly oral DMBA.
a,b,c: Shows the difference between the groups (values with different lower case letters show statistically significant differences, $p<0.001$).

No significant difference was present in the serum fluoride levels in the control group, the DMBA solvent (sesame oil) group, the 1 ppm fluoride group, the DMBA-treated group, and the 1 ppm fluoride+DMBA group ($p>0.05$). No significant difference was present in the serum fluoride levels in the 15 ppm fluoride group, the 30 ppm fluoride group, and the 15 ppm fluoride+DMBA group ($p>0.05$). No significant difference was present in the serum fluoride levels in the 30 ppm fluoride group and the 30 ppm fluoride+DMBA group ($p>0.05$). The serum fluoride level was significantly increased in the 15 ppm fluoride group and the 30 ppm fluoride group compared to the control group ($p<0.001$).The serum fluoride level was significantly increased in the 30 ppm fluoride+DMBA group compared to the 15 ppm fluoride+DMBA group ($p<0.001$).

4.3. Anxiety evaluation: In the intragroup analysis of the elevated plus maze test results concerning the time spent in the open field, on day 0 compared to the time on day 90, a significant decrease was observed in the DMBA vehicle, 1 ppm fluoride, DMBA, and 1 ppm fluoride+DMBA groups ($p<0.01$), in the 15 ppm fluoride group ($p<0.001$), and in the 15 ppm fluoride+DMBA group ($p<0.05$). A significant increase, in the time spent in the open field, on day 0 compared to the time on days

30, 90, was observed in the 30 ppm fluorine group ($p<0.01$) and the 30 ppm fluoride+DMBA group ($p<0.05$) (Table 1 and Figures 2A and 2B).

Table 1. Time spent in open field (s) (Std D=standard deviation, F=fluoride, DMBA=7,12 dimethylbenz(a)anthracene)

Group	Day 0 Mean+Std D	Day 30 Mean+Std D	Day 60 Mean+Std D	Day 90 Mean+Std D	p: →
Control group	20.18 ±2.97^{bc} BC	26.51 ±4.30^{b} BC	35.91 ±8.48^{a} A	22.80 ±4.68^{c} C	<0.05
DMBA solvent	23.89 ±2.99^{a} AB	28.67 ±5.02^{a} B	15.02 ±4.86^{b} C	12.11 ±4.09^{b} D	<0.01
1 ppm F	21.43 ±3.53^{a} ABC	18.53 ±4.10^{b} DEF	11.80 ±4.37^{c} CD	8.54 ±2.56^{c} D	<0.01
15 ppm F	20.32 ±4.21^{a} BC	16.71 ±4.81^{b} EF	8.83 ±2.04^{c} D	3.50 ±1.77^{d} E	<0.001
30 ppm F	26.82 ±3.14^{c} A	35.77 ±6.62^{b} A	38.78 ±3.73^{b} A	47.29 ±5.65^{a} A	<0.01
DMBA	16.82 ±6.08^{a} C	14.37 ±2.80^{ab} EF	12.71 ±2.81^{b} CD	9.12 ±2.64^{c} D	<0.01
DMBA +1 ppm F	20.32 ±3.80^{a} BC	12.39 ±6.76^{b} F	11.83 ±2.93^{b} CD	8.17 ±2.40^{c} D	<0.01
DMBA +15 ppm F	16.71 ±4.65^{b} C	20.49 ±9.30^{a} CDE	16.88 ±2.53^{b} C	12.43 ±2.37^{c} D	<0.05
DMBA +30 ppm F	23.10 ±10.94^{b} AB	23. 60 ±3.63^{b} BCD	24.95 ±5.04^{ab} B	28.58 ±7.99^{a} B	<0.05
p: ↓	<0.01	<0.001	<0.001	<0.001	

A, B, C, D, E, F: p: ↓ Shows difference between groups (in the same column values with different upper case letters show statistically significant differences).

a, b, c, d : p: → Shows the difference within the group (in the same line values with different lower case letters show statistically significant differences).

A significant increase ($p<0.001$) was detected in the 30 ppm fluoride-treated group at the 30th day in comparison with the control group. Significant decreases were present on the 30th day in the 1 ppm fluoride, 15 ppm fluoride, DMBA and 1 ppm

fluoride+DMBA groups, in comparison with the control group ($p < 0.001$). On the 60th day, compared to the control group, there were significant decreases ($p<0.001$) in all the groups, other than the 30 ppm fluoride group. On the 90th day, compared to the control group, inter-group comparisons showed significant increases in the 30 ppm fluorine and 30 ppm fluoride+DMBA groups ($p < 0.001$) while there were significant decreases in the other groups ($p<0.001$).

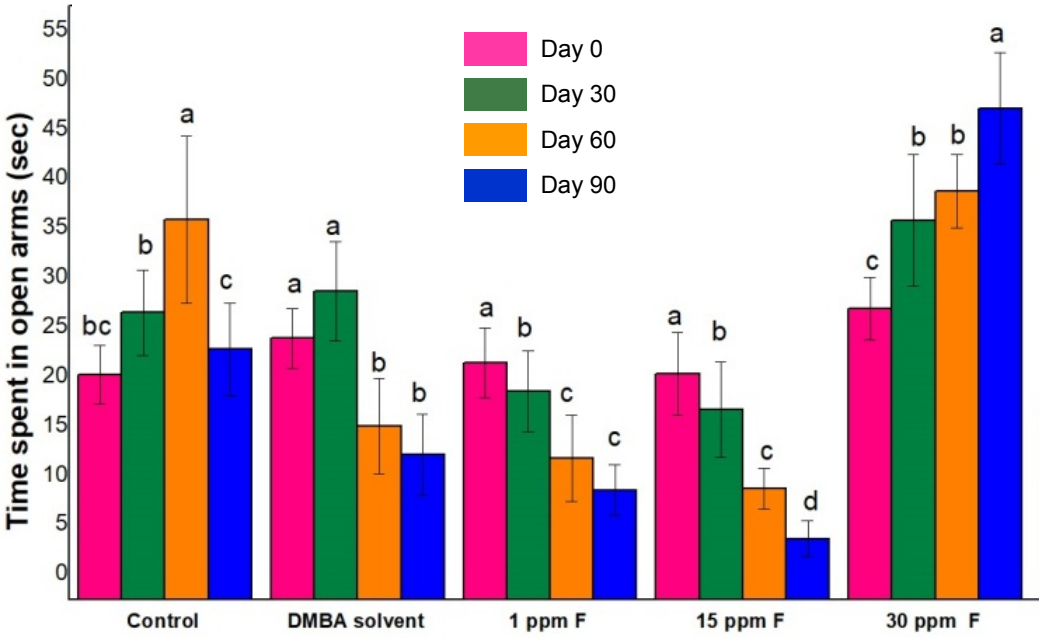

Figure 2A. Time spent in open field (s) for the control, DMBA solvent, and the 1, 15, and 30 ppm fluoride groups.
a, b, c, d: Shows the difference within the groups (values with different lower case letters show statistically significant differences are present within that group).

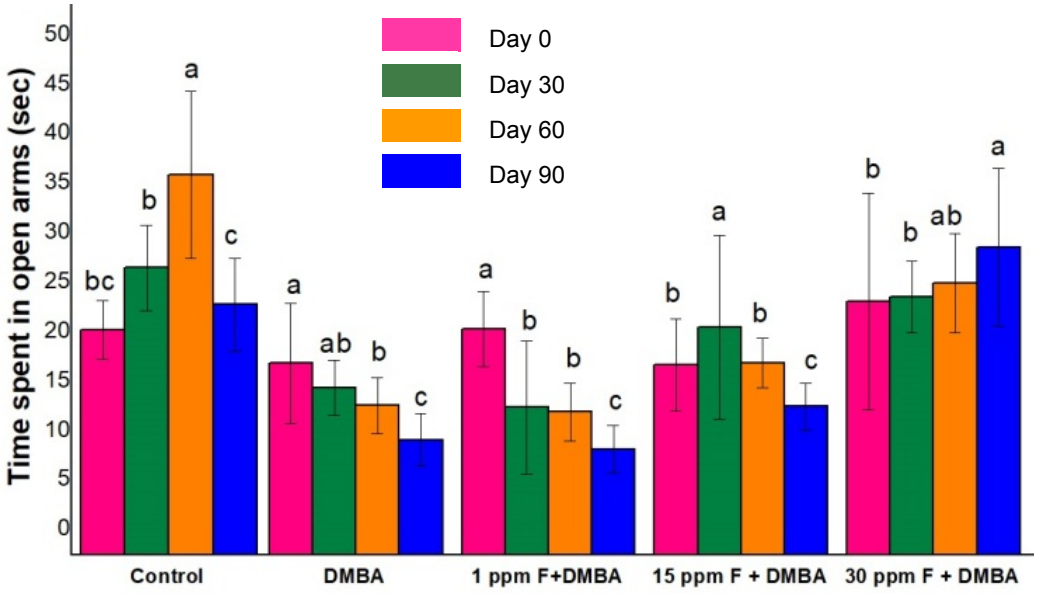

Figure 2B. Time spent in open field (s) for the control, DMBA, and the 1, 15, and 30 ppm fluoride+DMBA groups.
a, b, c, d: Shows the difference within the groups (values with different lower case letters show statistically significant differences are present within that group).

In the intragroup analysis of the number of open field entries on the elevated plus maze test, a decrease was present for all the groups on days 30, 60, and 90 compared to day 0. In the intergroup analysis of the number of open field entries on the elevated plus maze test, an increase was present on day 90 in the 30 ppm F group compared to the control group ($p<0.001$) (Table 2 and Figures 3A and 3B).

Table 2. Number entering into the open field (s) (Std D=standard deviation, F=fluoride, DMBA=7,12 dimethylbenz(a)anthracene)

Group	Day 0 Mean+Std D	Day 30 Mean+Std D	Day 60 Mean+Std D	Day 90 Mean+Std D	p: →
Control group	20.8 ±9.0^{a}	6.2 ±9.3^{b}	3.5 ±2.3^{b}	3.7 ±1.6^{b} B	<0.05
DMBA solvent	19.3 ±5.2^{a}	2.3 ±1.4^{b}	2.3 ±1.0^{b}	3.3 ±2.9^{b} B	<0.01
1 ppm F	19.3 ±10.8^{a}	1.7 ±0.5^{b}	2.5 ±2.0^{b}	1.6 ±1.1^{b} B	<0.01
15 ppm F	25.4 ±12.5^{a}	2.8 ±1.9^{b}	1.5 ±0.5^{b}	2.0 ±1.8^{b} B	<0.001
30 ppm F	22.1 ±7.5^{a}	4.3 ±4.6^{b}	5.8 ±5.8^{b}	9.6 ±8.8^{a} A	<0.01
DMBA	17.2 ±12.7^{a}	2.4 ±1.8^{b}	2.4 ±2.1^{b}	3.3 ±2.8^{b} B	<0.05
DMBA+1 ppm F	22.7 ±17.2^{a}	1.0 ±0.0^{b}	2.5 ±2.3^{b}	3.2 ±2.6^{b} B	<0.05
DMBA+15 ppm F	16.0 ±7.7^{a}	2.6 ±1.3^{b}	2.0 ±0.9^{b}	3.4 ±3.3^{b} B	<0.01
DMBA+30 ppm F	25.8 ±4.7^{a}	2.9 ±1.1^{c}	5.3 ±4.8^{bc}	6.1 ±2.0^{b} AB	<0.001
p: ↓	>0.05	>0.05	>0.05	<0.001	

A, B : p: ↓ Shows difference between groups (in the same column values with different upper case letters show statistically significant differences).

a, b, c : p: → Shows the difference within the group (in the same line values with different lower case letters show statistically significant differences).

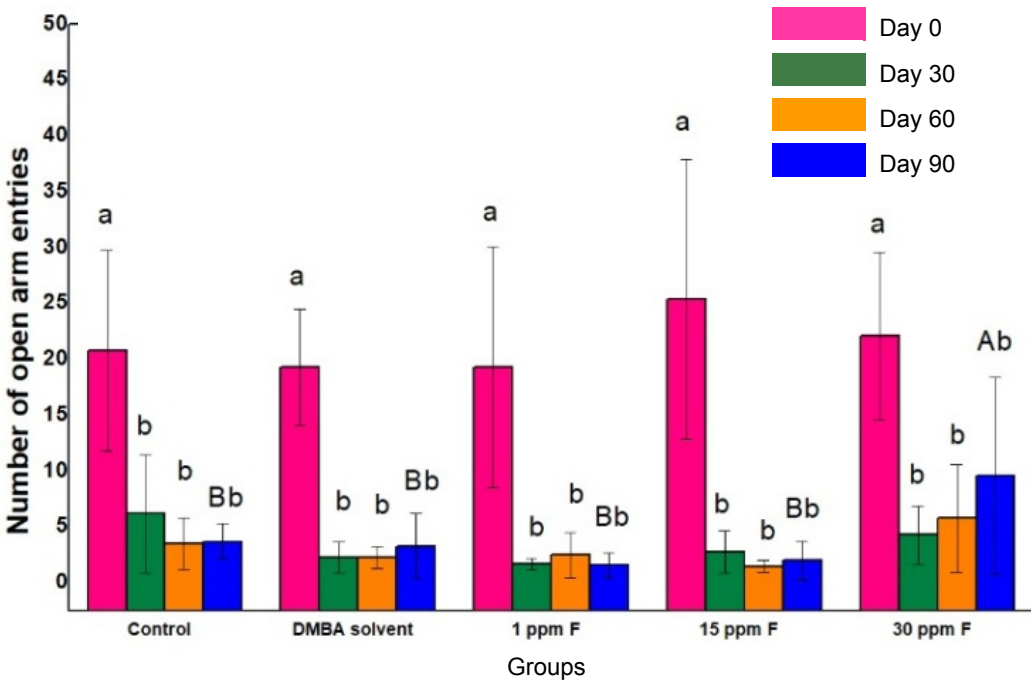

Figure 3A. Number entering into the open field for the control, DMBA solvent, and the 1, 15, and 30 ppm fluoride groups.
A, B: ↓ Shows the difference between groups (values with different upper case letters show statistically significant differences are present in the same column in different groups).
a, b, c: → Shows the difference within the groups (values with different lower case letters show statistically significant differences are present within that group).

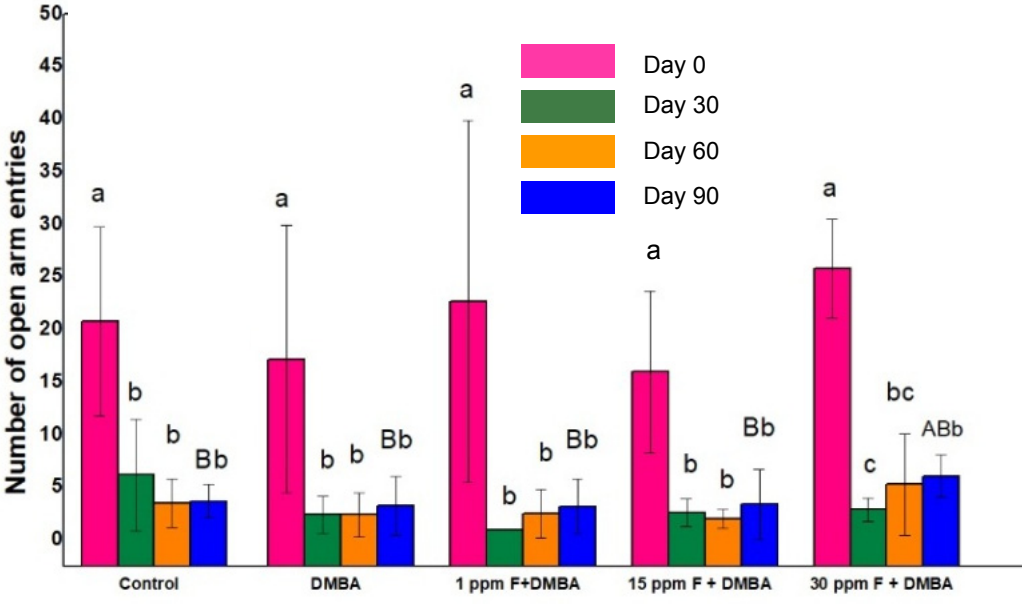

Figure 3B. Number entering into the open field for the control, DMBA, and the 1, 15, and 30 ppm fluoride+DMBA groups.
A, B: ↓ Shows the difference between groups (values with different upper case letters show statistically significant differences are present in the same column in different groups).
a, b, c: → Shows the difference within the groups (values with different lower case letters show statistically significant differences are present within that group).

4.4. Evaluation of motor activity: The walking durations on the rotarod are shown in Figures 4A and 4B

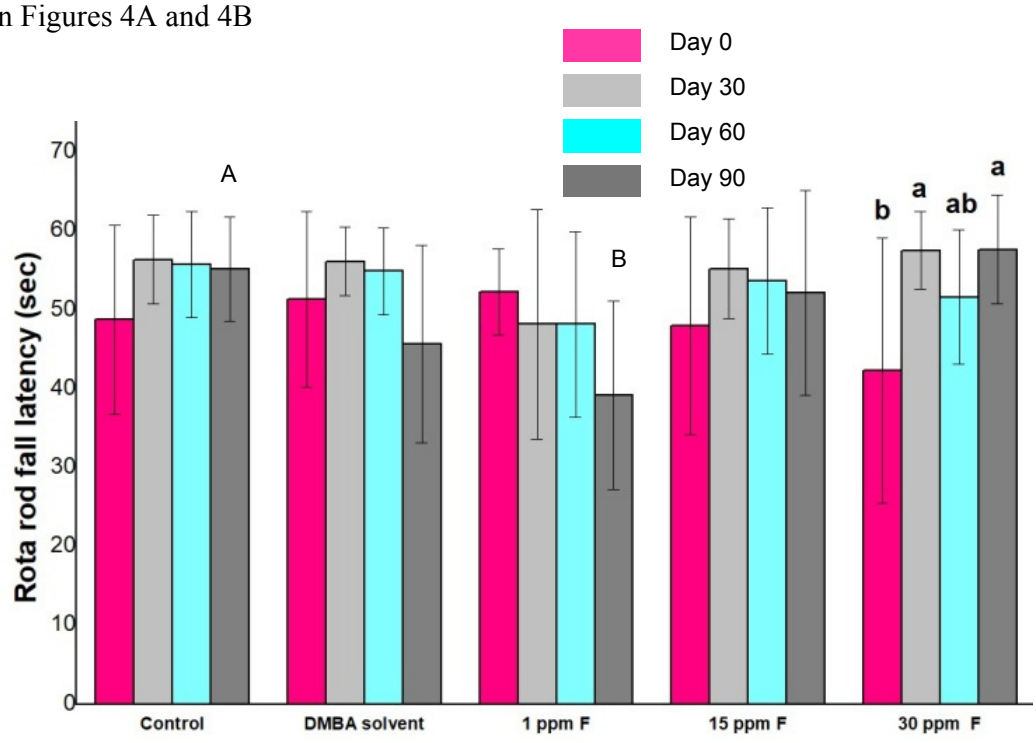

Figure 4A. Walking duration on the rotarod (sec).
a, b, c: → Shows the difference within the groups (values with different lower case letters show statistically significant differences are present within that group, $p<0.05$).

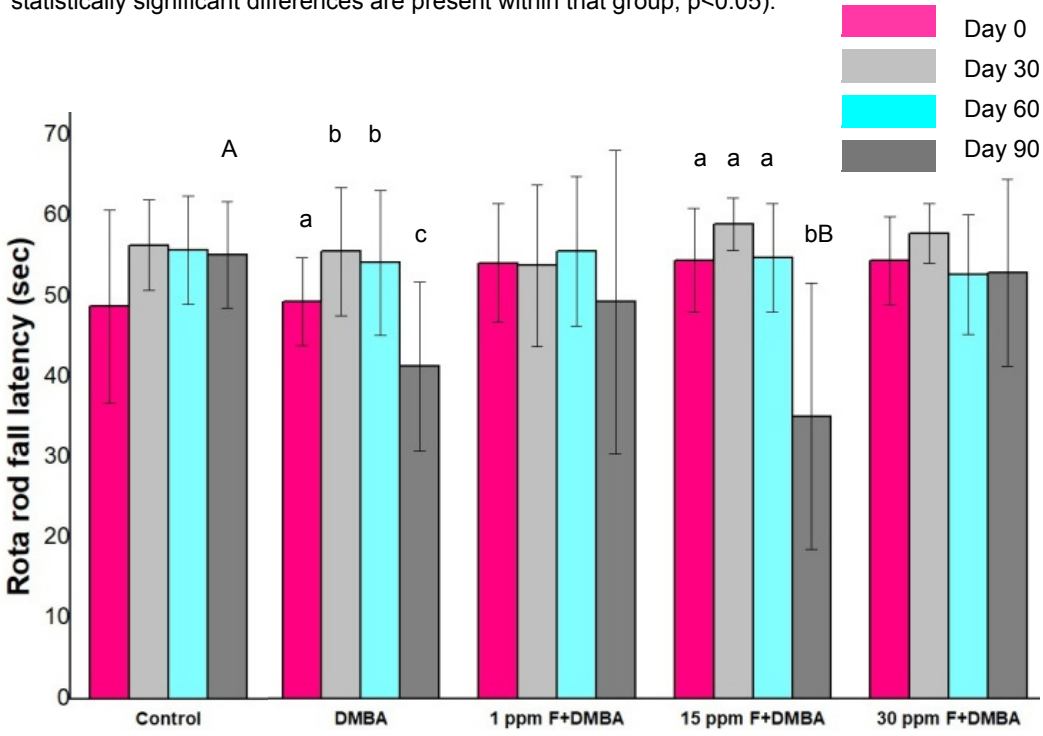

Figure 4B. Walking duration on the rotarod (sec). a, b, c: → Shows the difference within the groups (values with different lower case letters show statistically significant differences are present within that group, $p<0.05$). A, B: ↓ Shows the difference between groups (values with different upper case letters show statistically significant differences are present in the same column in different groups, $p<0.05$).

In the intragroup comparisons of the motor activity performed with the rotarod test, it was found that in the 30 ppm fluorine group, the walking duration time on the rotating rod was increased on days 30 and 90 compared to day 0 ($p<0.05$).

In the DMBA group, compared to day 0, the mean time increased on days 30 and 60 ($p <0.05$) and decreased on day 90 ($p<0.05$). In the 15 ppm F+DMBA, compared to day 0, the mean time decreased on day 90 ($p<0.05$).

In the comparisons between the groups, the walking time decreased in the 1 ppm fluorine and 15 ppm F+ DMBA groups ($p<0.05$) compared to the control group

4.5. Learning and Memory Performances: In this study, the Morris water tank test was applied to the rats for four consecutive days commencing on day 0 and at the end of the 1st, 2nd, and 3rd months. With this test, the effects of DMBA and fluoride on learning were examined and the time (sec) for the animals to find the hidden platform were evaluated.

As shown in Figure 5 and Table 3, the time taken for the rats in all the groups to find the platform, in the four consecutive days of trials commencing on day 0, was significantly less in the trials on day 4 than in the trials on day 1. The day 0 trials were performed without the rats being given any substances (fluoride or DMBA) and no intergroup comparisons were made.

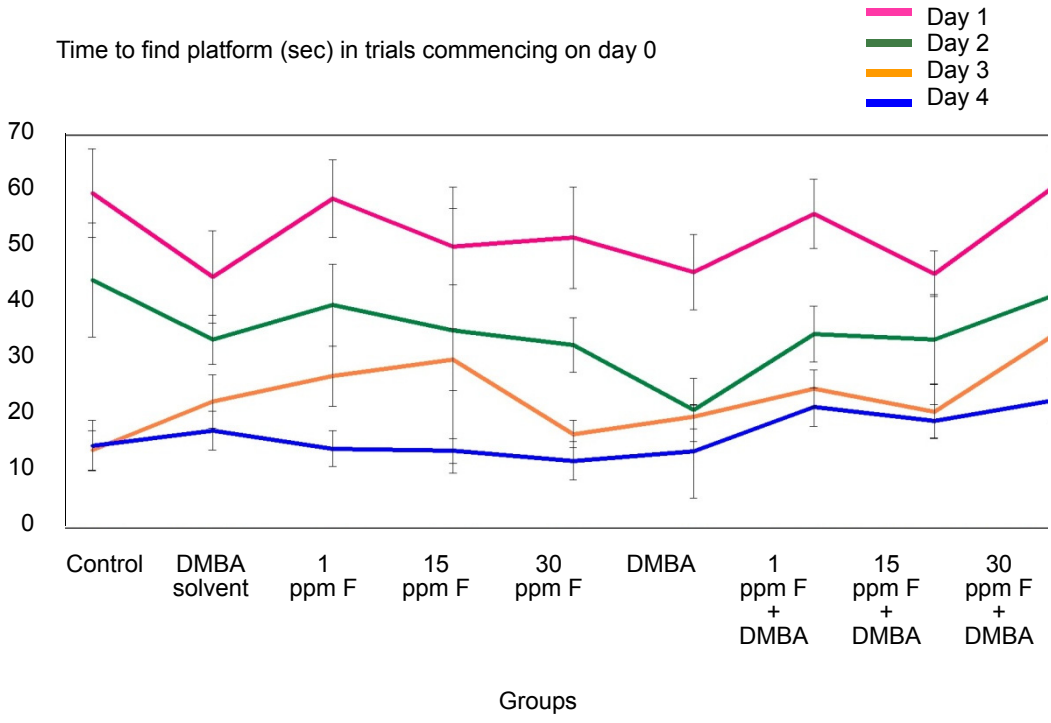

Figure 5. Platform finding time of rats to find platform (sec) in the four consecutive days of trials commencing on day 0.

Table 3. Platform finding time (sec) of the rats in the four consecutive days of trials commencing on day 0 (Std D=standard deviation)

Group	Platform finding time (sec)				p: →
	Day 1 (Mean ±Std D)	Day 2 (Mean ±Std D)	Day 3 (Mean ±Std D0	Day 4 (Mean ±Std D)	
Control group	59.8 ±7.9[a]	44.3 ±10.2[b]	13.9 ±3.5[c]	14.7 ±4.5[c]	<0.001
DMBA solvent	44.8 ±8.2[a]	33.6 ±4.40[b]	22.6 ±4.8[c]	17.4 ±3.5[d]	<0.001
1 ppm F	58.8 ±6.9[a]	39.8 ±7.30[b]	27.1 ±5.4[b]	14.2 ±3.2[c]	<0.001
15 ppm F	50.2 ±6.8[a]	35.3 ±25.50[b]	30.1 ±5.5[b]	13.8 ±2.2[c]	<0.01
30 ppm F	51.8 ±9.0[a]	32.7 ±4.90[b]	16.8 ±2.4[c]	12.0 ±3.4[c]	<0.001
DMBA	45.7 ±6.7[a]	21.1 ±5.60[b]	19.9 ±2.2[b]	13.7 ±8.3[c]	<0.01
DMBA+1 ppm F	56.1 ±6.2[a]	34.6 ±5.00[b]	24.9 ±3.3[c]	21.6 ±3.4[c]	<0.001
DMBA+15 ppm F	45.4 ±4.1[a]	33.7 ±8.00[b]	20.8 ±4.8[c]	19.1 ±3.0[c]	<0.01
DMBA+30 ppm F	60.9 ±7.2[a]	41.6 ±7.20[b]	34.5 ±5.2[c]	22.8 ±3.8[d]	<0.001

a, b, c, d : p: → Shows the difference within the group (in the same line values with different lower case letters show statistically significant differences).

In the Morris Water Tank trials commencing on the 30th day, the time taken for the rats in all the groups to find the platform, in the four consecutive days of trials was significantly less in the trials on day 4 than in the trials on day 1. (Table 4 and Figure 6).When the time taken to find the platform between the groups was compared, it was found that there were statistically significant differences between the control group and the DMBA, 1 ppm F+DMBA, and 15 ppm F+DMBA groups on days 1, 2, 3, and 4 ($p<0.001$).

Table 4. Platform finding time (sec) of the rats in the four consecutive days of trials commencing on day 30, the end of the 1st month (Std D=standard deviation)

Group	Platform finding time (sec)				p: →
	Day 1 (Mean ±Std D)	Day 2 (Mean ±Std D)	Day 3 (Mean ±Std D)	Day 4 (Mean ±Std D)	
Control group	24.3 ±6.4[a] B	14.5 ±3.2[b] D	8.4 ±2.2[c] D	6.0 ±2.1[c] C	<0.01
DMBA solvent	23.4 ±3.1[a] B	14.7 ±3.3[b] D	6.6 ±1.8[c] D	7.9 ±1.6[c] C	<0.001
1 ppm F	29.5 ±3.8[a] B	14.8 ±3.6[b] D	8.9 ±2.1[c] D	7.5 ±1.3[c] C	<0.001
15 ppm F	25.0 ±4.5[a] B	16.4 ±3.9[b] CD	9.2 ±3.1[c] D	7.6 ±1.5[c] C	<0.001
30 ppm F	20.5 ±4.3[a] B	13.1 ±3.9[b] D	8.2 ±1.6[c] D	7.0 ±1.2[c] C	<0.001
DMBA	38.5 ±5.0[a] A	20.9 ±3.8[b] BC	19.5 ±8.4[b] B	15.2 ±2.4[c] A	<0.01
DMBA+1 ppm F	40.6 ±22.1[a] A	24.9 ±3.4[b] AB	23.9 ±3.8[b] A	16.1 ±3.8[c] A	<0.05
DMBA+15 ppm F	45.7 ±7.8[a] A	26.0 ±7.1[b] A	18.0 ±3.7[c] BC	13.0 ±10.5[c] AB	<0.01
DMBA+30 ppm F	27.1 ±3.9[a] B	22.2 ±4.3[b] AB	14.3 ±2.4[c] C	8.9 ±1.4[d] BC	<0.001
p: ↓	<0.001	<0.001	<0.001	<0.001	

A, B, C, D : p: ↓ Shows the difference between the groups (in the same column values with different upper case letters show statistically significant differences).

a, b, c, d : p: → Shows the difference within the group (in the same line values with different lower case letters show statistically significant differences).

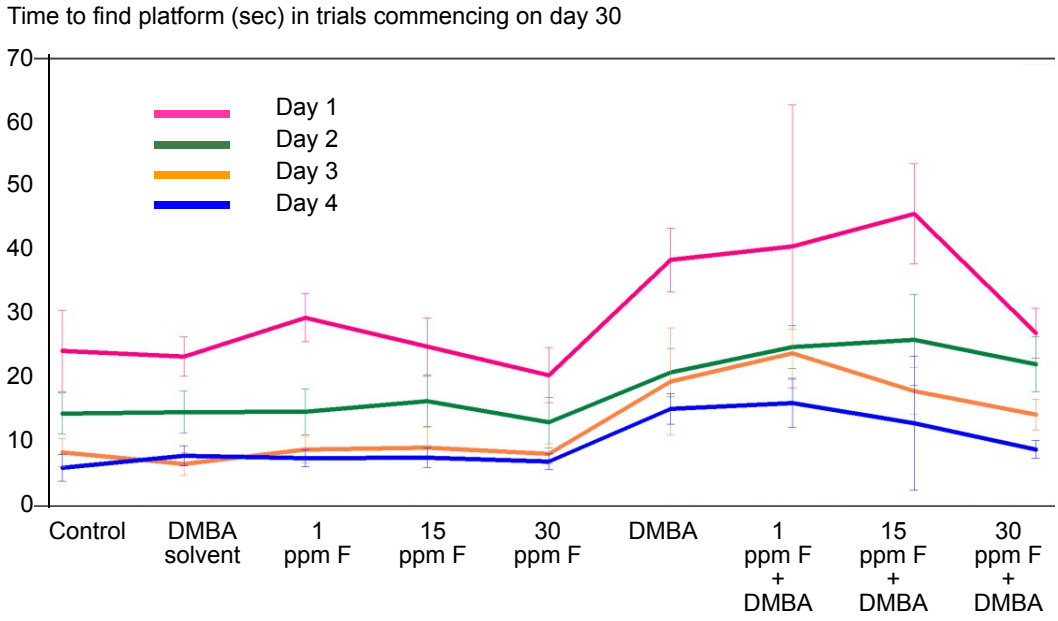

Figure 6. Platform finding time of rats to find platform (sec) in the four consecutive days of trials commencing on day 30.

In the Morris water tank trials performed on the 60th day, it was determined that the time taken for the rats to find the platform decreased significantly on the 4th experimental day compared to the 1st experimental day in all the groups [control ($p < 0.05$), DMBA solvent ($p<0.01$), 1 ppm F ($p<0.01$), 15 ppm ($p < 0.05$), 30 ppm F ($p < 0.001$), DMBA ($p<0.05$), 1 ppm F+DMBA ($p < 0.05$), 15 ppm F+DMBA ($p<0.01$), and 30 ppm F+DMBA ($p<0.01$) groups] (Figure 7 and Table 5).

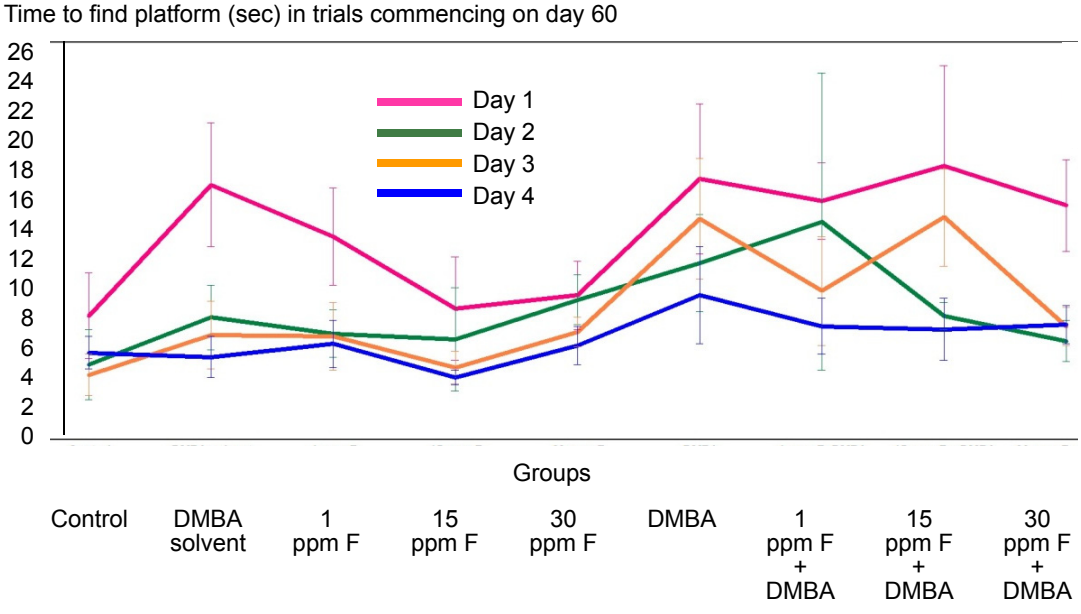

Figure 7. Platform finding time of rats to find platform (sec) in the four consecutive days of trials commencing on day 60.

Table 5. Platform finding time (sec) of the rats in the four consecutive days of trials commencing on day 60, the end of the 2nd month (Std D=standard deviation)

Group	Platform finding time (sec)				p: →
	Day 1 (Mean ±Std D)	Day 2 (Mean ±Std D)	Day 3 (Mean ±Std D)	Day 4 (Mean ±Std D)	
Control group	8.4 ±2.9^{a} D	5.1 ±2.4^{b} C	4.4 ±1.4^{c} D	5.9 ±1.1^{b} BCD	<0.05
DMBA solvent	17.3 ±4.2^{a} AB	8.3 ±2.2^{b} BC	7.1 ±2.3^{b} CD	5.6 ±1.4^{c} CD	<0.01
1 ppm F	13.8 ±3.3^{a} BC	7.2 ±1.6^{b} C	7.0 ±2.3^{b} CD	6.5 ±1.6^{b} BC	<0.01
15 ppm F	8.9 ±3.5^{a} D	6.8 ±3.5^{b} C	4.9 ±1.1^{c} CD	4.2 ±0.5^{c} D	<0.05
30 ppm F	9.8 ±2.3^{a} CD	9.5 ±1.7^{a} BC	7.3 ±1.0^{b} C	6.4 ±1.3^{b} BC	<0.001
DMBA	17.7 ±5.1^{a} AB	12.0 ±3.3^{c} AB	15.0 ±4.1^{b} A	9.8 ±3.3^{d} A	<0.05
DMBA+1 ppm F	16.2 ±2.6^{a} AB	14.8 ±10.1^{b} A	10.1 ±3.7^{c} B	7.7 ±1.9^{d} B	<0.05
DMBA+15 ppm F	18.6 ±6.8^{a} A	8.4 ±0.9^{c} BC	15.1 ±3.3^{b} A	7.5 ±2.1^{c} BC	<0.01
DMBA+30 ppm F	15.9 ±3.1^{a} AB	6.7 ±1.4^{c} C	7.7 ±1.3^{b} BC	7.8 ±1.3^{b} B	<0.01
p: ↓	<0.001	<0.001	<0.001	<0.001	

A, B, C, D : p: ↓ Shows the difference between the groups (in the same column values with different upper case letters show statistically significant differences).

a, b, c, d : p: → Shows the difference within the group (in the same line values with different lower case letters show statistically significant differences).

When the time to find the platform between the groups was compared, in the Morris water tank trials performed on the 60th day, the platform finding time was significantly increased, on day 1, in the 1 ppm F group compared to the control group ($p<0.001$); on day 2 in the 1 ppm F+DMBA group compared to the control, the DMBA solvent, and the 1 ppm F groups ($p<0.001$), on day 3, in the DMBA, 1 ppm F+DMBA, and 15 ppm F+DMBA groups compared to the control, DMBA solvent, 1ppm F, and 15 ppm F groups ($p<0.001$). and, on day 4, in the DMBA group compared to the DMBA solvent group ($p<0.001$).

In the Morris water tank trials conducted on the 90th day, the time taken for the rats to find the platform decreased significantly from the first experimental day to the fourth experimental day in all the groups [control ($p<0.01$), DMBA solvent ($p<0.01$), 1 ppm F ($p <0.001$), 15 ppm ($p <0.001$), 30 ppm F ($p <0.001$), DMBZ ($p<0.01$), 1 ppm F+DMBA ($p<0.05$), 15 ppm F+DMBA ($p<0.05$), and 30 ppm F+DMBA ($p<0.05$) groups] (Figure 8 and Table 6).

When the time to find the platform between the groups was compared in the trials commencing on the 90th day, it was found that the platform finding time, on the first experimental day increased significantly in the 15 ppm F group compared the control group ($p <0.01$).

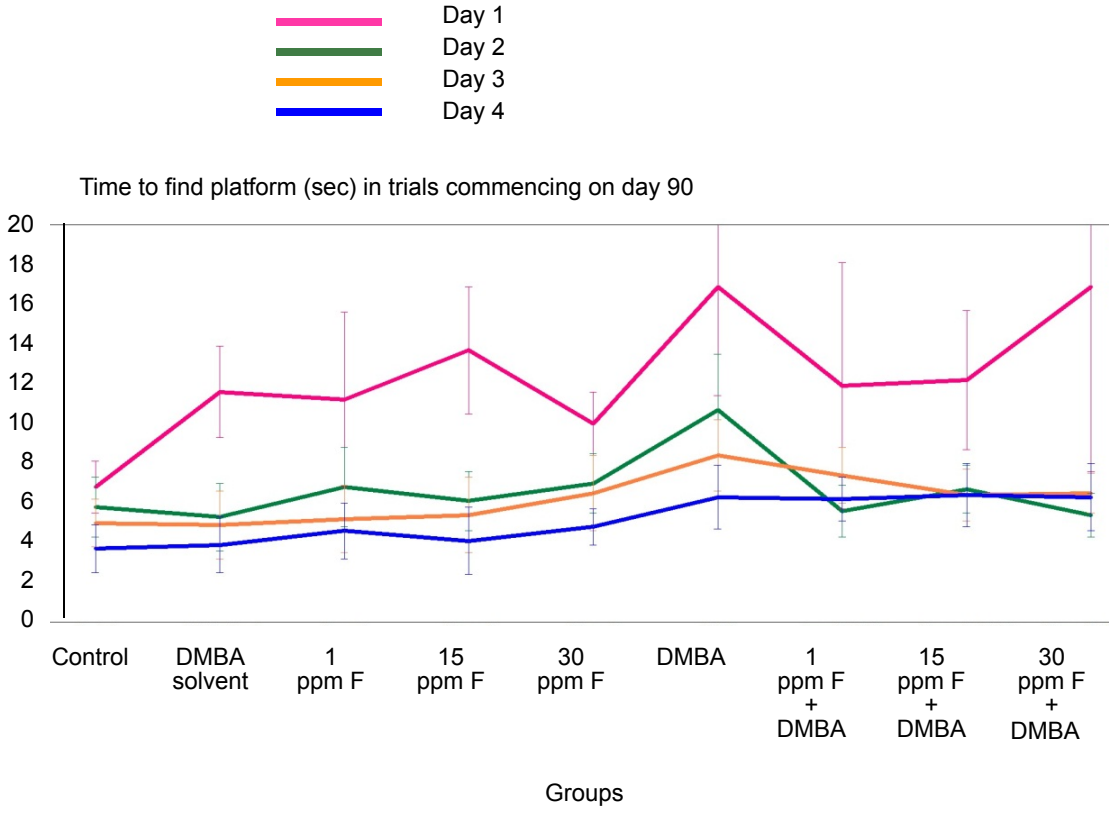

Figure 8. Platform finding time of rats to find platform (sec) in the four consecutive days of trials commencing on day 90.

Table 6. Platform finding time (sec) of the rats in the four consecutive days of trials commencing on day 90, the end of the 3rd month (Std D=standard deviation)

Group	Platform finding time (sec)				p: →
	Day 1 (Mean ±Std D)	Day 2 (Mean ±Std D)	Day 3 (Mean ±Std D)	Day 4 (Mean ±Std D)	
Control group	6.8 ±1.3[a] C	5.8 ±1.5[b] B	5.0 ±1.2[c] C	3.7 ±1.2[d] C	<0.01
DMBA solvent	11.6 ±2.3[a] ABC	5.3 ±1.7[b] B	4.9 ±1.7[b] C	3.9 ±1.4[c] C	<0.01
1 ppm F	11.2 ±4.4[a] BC	6.8 ±2.0[b] B	5.2 ±1.7[b] C	4.6 ±1.4[b] BC	<0.001
15 ppm F	13.7 ±3.2[a] AB	6.1 ±1.5[b] B	5.4 ±1.9[b] C	4.1 ±1.7[b] C	<0.001
30 ppm F	10.0 ±1.6[a] BC	7.0 ±1.5[b] B	6.5 ±1.9[b] ABC	4.8 ±0.9[b] ABC	<0.001
DMBA	16.9 ±5.5[a] A	10.7 ±2.8[b] A	8.4 ±1.8[c] A	6.3 ±1.6[d] A	<0.01
DMBA+1 ppm F	11.9 ±6.2[a] ABC	5.6 ±1.3[c] B	7.4 ±1.4[b] AB	6.2 ±1.1[c] AB	<0.05
DMBA+15 ppm F	12.2 ±3.5[a] ABC	6.7 ±1.2[b] B	6.4 ±1.3[b] BC	6.4 ±1.6[b] A	<0.05
DMBA+30 ppm F	16.9 ±9.3[a] A	5.4 ±1.1[b] B	6.5 ±1.0[b] ABC	6.3 ±1.7[b] AB	<0.05
p: ↓	<0.01	<0.001	<0.01	<0.001	

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When, on the 90th day Morris water tank trials, the rats swam for one minute in the platform-free maze, the time spent in the platform quadrant was significantly decreased, on day 4, in the DMBA group compared to the control group ($p<0.05$) (Figure 9 and Table 7).

When, in the Morris water tank trials conducted on the 90th day, the rats swam for one minute in the platform-free maze, the time spent in the platform quadrant decreased significantly from the first experimental day to the fourth experimental day in the 30 ppm F+DMBA group ($p<0.05$).

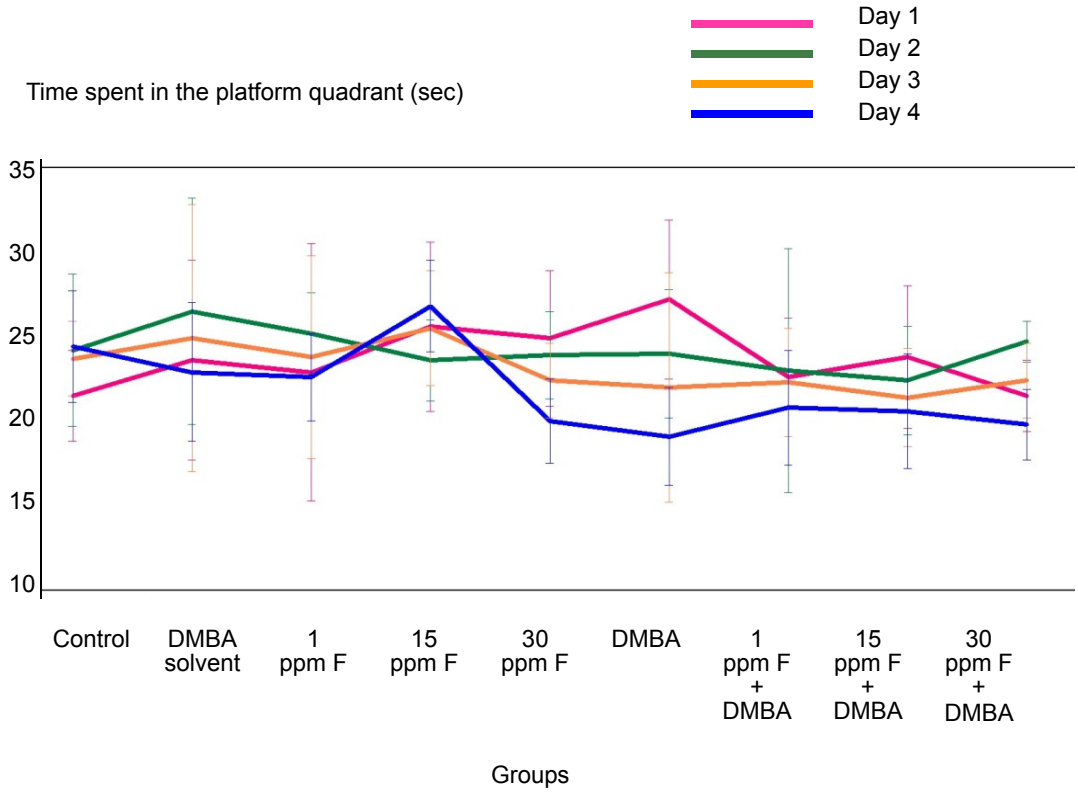

Figure 9. Time spent by the rats in the platform area (sec) in the four consecutive days of trials commencing on day 90.

Table 7. Time spent in the platform area (sec) of the rats in the four consecutive days of trials commencing on day 90, the end of the 3rd month (Std D=standard deviation)

Group	Time spent in the platform area (sec)				p: →
	Day 1 (Mean ±Std D)	Day 2 (Mean ±Std D)	Day 3 (Mean ±Std D)	Day 4 (Mean ±Std D)	
Control group	21.54 ±2.68	24.24 ±4.51	23.65 ±2.19	24.40 ±3.34 AB	>0.05
DMBA solvent	23.56 ±5.86	26.47 ±6.69	24.94 ±7.91	22.89 ±4.1 BC	>0.05
1 ppm F	22.91 ±7.59	25.24 ±2.40	23.77 ±5.95	22.59 ±2.64 BC	>0.05
15 ppm F	25.64 ±4.98	23.64 ±2.42	25.51 ±3.39	26.75 ±2.72 A	>0.05
30 ppm F	24.88 ±3.99	23.94 ±2.64	22.42 ±2.25	19.96 ±2.50 C	>0.05
DMBA	27.23 ±4.68	24.02 ±3.85	21.96 ±6.77	19.07 ±2.92 C	>0.05
DMBA+1 ppm F	22.57 ±3.49	22.99 ±7.23	22.31 ±3.18	20.84 ±3.45 BC	>0.05
DMBA+15 ppm F	23.76 ±4.25	22.40 ±3.22	21.37 ±2.87	20.62 ±3.45 BC	>0.05
DMBA+30 ppm F	21.52 ±2.12^{b}	24.72 ±1.24^{a}	22.41 ±2.21^{ab}	19.76 ±2.05^{c} C	<0.05
p: ↓	>0.05	>0.05	>0.05	<0.05	

A, B, C, D : p: ↓ Shows the difference between the groups (in the same column values with different upper case letters show statistically significant differences).

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4. DISCUSSION

Since the nervous system controls all physiological processes in the organism, a wide variety of symptoms may occur when toxicity occurs. In addition to tremors accompanied by a loss of coordination, motor disorders, ranging from paralysis to convulsions, may develop. Psychological changes such as excitation, depression, and irritability may occur. Cognitive disorders such as memory loss, confusion, speech disorders, and learning disability can also occur. The high rate of polycyclic aromatic hydrocarbon pollution in the regions where endemic fluorosis is also seen adversely affects the quality of life of people and animals living in these regions and increases the risk of chronic poisoning.

According to the results of our literature reviews, the high fluorine concentration in water and soil has been targeted as the main cause of health problems in humans and animals living in volcanic areas where endemic fluorosis is experienced. On the other hand, the negative effects of exposure to polycyclic aromatic hydrocarbon derivatives together with fluorine have been ignored. In this study, we investigated how anxiety, motor activity, and learning can be affected in rats exposed to fluorine and DMBA, a polycyclic aromatic hydrocarbon derivative. In our study, rats were treated with fluorine at different concentrations (1, 15, and 30 ppm) in drinking water for 90 days. At the end of the 90th day, the fluorine was stopped and the blood levels of fluorine were exhumed in blood collected from the rats by the intracardiac route. The results of the analysis showed that the serum fluorine levels increased significantly in the rats exposed to increased concentrations of fluorine.

The elevated plus maze test is one of the methods used to evaluate the anxiety behaviors of experimental animals. In this anxioselective test, anxiolytic substances have been shown to increase the frequency of entering into open arms and the time period passed in open arms. Anxiogenic substances have been reported to cause the opposite effects and decrease such parameters.[31] In the present study, the effects of fluorine and DMBA on anxiety were evaluated in the elevated plus maze test. For the behavioral evaluations of anxiety, the time spent in open arms and the number of entries into the open arms were evaluated.

On day 90, fluorine in a concentration of 30 ppm and 30 ppm F+DMBA significantly increased, compared to the control groups, the duration of the time spent in the open arms in the anxiety trials with the elevated plus maze test (Table 1). In addition, the number of open arm entries significantly increased in the 30 ppm F group compared to the control group (Table 2).

These results suggest that fluorine can produce anxiolytic effect at 30 ppm rather than DMBA. In addition, on day 90, fluorine in a concentration of 1 ppm and 15 ppm significantly decreased, compared to the control group, the duration of the time spent in the open arms in the anxiety trials with the elevated plus maze test, suggesting that 1 and 15 ppm of F may be anxiogenic (Table 1).

The results of scientific studies indicate that the gamma amino butyric acid (GABA) benzodiazepine receptor-Cl-ionophore complex, the noradrenergic system, and the serotonergic system have important roles in the formation and maintenance of both normal conditions and pathological anxiety.[32,33] In addition to neurotransmitter systems, dopaminergic neurons and cholinergic neurons contribute

to the development of anxiety by increasing stimulation and attention, whereas antidopaminergic and anticholinergic drugs do not have a significant anxiolytic effect. It is known that hypothalamohypophyseal pathways and ACTH are also inactive in depressive patients and increase activity in anxiety and increased stress.

In addition, central neuropeptides, such as cholecystokinin and substance P, inhibitory neurotransmitters in the central nervous system other than GABA, and the adenosine and glutamatergic systems together with central nitric oxide (NO) play a role in the development and maintenance of anxiety.[33,34] Additional studies are needed to fully elucidate the anxiolytic effect of DMBA and low-dose fluorine, or the anxiolytic effect of high-dose fluorine (30 ppm). In addition, a detailed analysis of neurotransmitter levels or other factors that may be effective in anxiety is needed.

Fluorine can cause functional and biochemical changes in the nervous system by crossing the blood brain barrier.[35] If the fluorine is taken in high doses, problems occur in the musculoskeletal system. The basis of the rotarod test is that the disruption of the dopaminergic system will lead to a reduction in motor skills. In an experimental study on rats, 5 mg /kg of fluorine administered 5 times a week showed no effect on motor activity in the rotarod test at 6 weeks.[36] Ekambaram and Paul[37] administered 500 ppm NaF for 60 days and set up a fluorosis experiment on rats. They observed a significant decrease in locomotor activity in rats according to rotarod test results. PAH derivatives have been reported to cause decreased neurophysiological responses, autonomic functions, and motor activity due to increased concentrations in the brain after acute exposure.[38]

In this study, an increase in the walking duration on the rotarod test occurred with 30 ppm F on days 30 and 90, compared to day 0, and with DMBA on days 30 and 60, compared to day 0,whereas a decrease in walking duration occurred with DMBA, on day 90, compared to day 0, and with 15 ppm F+DMBA on day 90, compared to day 0. Our results related with effect of fluorine on motor activity are contradictory to those in the literature.[36,37] On the other hand, our results on the effect of DMBA on motor activity are parallel to those in the literature.[38] These results suggest an attenuation of acetylcholine levels thus leading a shift in the ACh/dopamine balance to the dopamine side. This situation may end up with an increased motor activity. The underlying cause of DMBA's reduction in motor activity may be due to damage to both the cholinergic and dopaminergic systems. It has been shown by different animal experiments that high amounts of fluorine cross the blood brain barrier, accumulate in the brain, create free radical damage, and subsequently adversely affect learning and memory.[39,40] Liu et al.[41,42] reported that the central nervous system neurotransmitter nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs) were suppressed by chronic fluorosis in human and animal experiments. In his study, Varner[43] reported a decrease in nicotinic acid receptors in animals drinking 1 ppm fluorine water, attenuation of the antioxidant defense system, and damage to the hippocampal region. An experimental study by Long et a.l[44] found that nicotinic acetylcholine receptors (nAChRs) are suppressed when the rats are treated with fluorine at 30 and 100 ppm. This result suggests that problems observed in fluorosis, such as learning deficits and attenuated IQ, may be mainly related to a decrease in acetylcholine.

On the other hand, information about the neurotoxic effects of PAH derivatives is quite limited. Benzo(a)pyrene exposure in humans causes attenuation in cognitive functions due to monoamine, amino acid and choline deficiency[45] and benzo(a)pyrene has been shown, in different studies, to cause adverse effects on neurobehavioral functions in increasing doses.[46] These studies are explained by changes in the expression of NR1 and NR2A subtypes of NMDA glutamate receptors in the brain region associated with memory and anxiety. The results shows that PAH derivatives may alter neurobehavioral functioning.[47]

In this study, in the Morris water tank test, it was observed that the hidden platform learning performance was found to be increased in all groups (Tables 4–6) whereas the memory performance was decreased in the 30 ppm F+DMBA (Table 7). In the comparisons between groups, it was observed that the duration for learning the platform location was prolonged with several of the DMBA and fluorine+DMBA groups (e.g, day 30, 4th day, DMBA, 1 ppm F+DMBA, and 30 ppm F+DMBA, compared to the control groups (Table 4); day 60, 3rd day, DMBA, 1 ppm F+DMBA, and 15 ppm F+DMBA, compared to the control groups, 4th day, DMBA compared to the DMBA solvent group (Table 5); and day 90, 3rd day, DMBA compared to the control groups (Table 6). These results showed a decrease in the learning performance. In the recall test, we evaluated time in which the rats swam in the quadrant where the platform should have been. In the target quadrant, a decrease occurred in the 30 ppm fluorine group, compared to the control group (Table 7). These results are consistent with studies suggesting that cognitive functioning is reduced by both PAH derivatives[45,46] and fluorine.[48,49] Our results suggest that fluorine and DMBA, in a toxic dose, prevent a permanent learning process in memory. In our study, the possible reason underlying the reduction of cognitive functions by fluorine and DMBA might be due to a possible reduction in the activity of cholinergic system.

CONCLUSIONS

The results of our study showed the toxins fluorine and DMBA, which are especially found in volcanic areas, can increase the risk of neurotoxic effects by decreasing learning and permanent memory. Motor activity was increased by fluorine and decreased by a combination of F+DMBA. DMBA could either increase or decrease motor activity depending on the time of assessment. In addition, F had an anxiogenic effect in a low dose and an anxiolytic activity at a high dose.of fluorine increases in a dose dependent fashion whereas DMBA can cause anxiety. According to these results, it is planned to clarify, in future studies, the mechanisms underlying the neurotoxic effects of fluorine and DMBA.

CONFLICT OF INTEREST: DECLARATION

The authors declare that they have no conflicts of interest concerning this article.

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